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Identification of Sibling Species of the Bryozoan *Bugula neritina* That Produce Different Anticancer Bryostatins and Harbor Distinct Strains of the Bacterial Symbiont "*Candidatus* Endobugula sertula"

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Abstract. Although the cosmopolitan marine bryozoan *Bugula neritina* is recognized as a single species, natural products from this bryozoan vary among populations. *B. neritina* is the source of the anticancer drug candidate bryostatin 1, but it also produces other bryostatins, and different populations contain different bryostatins. We defined two chemotypes on the basis of previous studies: chemotype O contains bryostatins with an octa-2,4-dienoate substituent (including bryostatin 1), as well as other bryostatins; chemotype M lacks bryostatins with the octa-2,4-dienoate substituent. *B. neritina* contains a symbiotic γ-proteobacterium "*Candidatus* Endobugula sertula," and it has been proposed that bryostatins may be synthesized by bacterial symbionts. In this study, *B. neritina* populations along the California coast were sampled for genetic variation and bryostatin content. Colonies that differ in chemotype also differ genetically by 8% in the mitochondrial cytochrome *c* oxidase subunit 1 (CO 1) gene; this difference is sufficient to suggest that the chemotypes represent different species. Each species contains a distinct strain of "*E. sertula*" that differs at four nucleotide sites in the small subunit ribosomal RNA (SSU rRNA) gene. These results indicate that the chemotypes have a genetic basis rather than an environmental cause. Gene sequences from an Atlantic sample matched sequences from the California chemotype M colonies, suggesting that this type may be cosmopolitan due to transport on boat hulls.

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Introduction

The recognition of sibling species throughout the world oceans has gained attention in recent years (Knowlton, 1993; Knowlton and Jackson, 1994; Muricy *et al.*, 1996; Palumbi, 1996). The identification of species that are difficult to distinguish by morphology alone is clearly important to understanding the ecology of a system. This has been demonstrated in studies of the coral *Montastraea annularis* and symbiotic dinoflagellates, which until recently were treated as a single species of coral and a single symbiont (Knowlton *et al.*, 1997; Knowlton *et al.*, 1992). Distinguishing these species is essential to understanding patterns of bleaching and interpreting data regarding environmental degradation and global climate changes (Knowlton *et al.*, 1992; Rowan *et al.*, 1997). In designing research programs, distinguishing sibling species is critical to understanding the biology of a system since these species can differ in their physiology and behavior in ways that affect their relationships and geographic distributions. If multiple species are treated as one species, we are likely to misinterpret the cause of observed patterns. Secondary metabolite chemistry is another character that probably varies among sibling species. The ability to distinguish closely related but apparently identical marine invertebrate species is critical to bioprospecting for pharmaceuticals from the sea and understanding the chemical ecology of the community. One of the obstacles in efforts to find potential drugs is the inconsistent recovery of compounds from different collections of the same organism. This has proven especially troublesome in work with sponges, and for certain species of sponges there is genetic evidence of extensive cryptic speciation (Muricy *et al.*, 1996). Christophersen attributed geographic variation

in bioactive compounds from some bryozoans to environmental factors (Christophersen, 1991). However, in this study we show that a variation in chemistry from the common marine bryozoan *Bugula neritina* is most likely due to genetic differences.

B. neritina is a common member of fouling communities on boat docks, boat hulls, and submerged rocks. This species has been treated as a single species throughout its cosmopolitan temperate range, although there are consistent chemistry differences among populations (Pettit, 1991). Some features of the biology of *B. neritina* could promote divergence between populations separated by long distances. The dispersal of the larvae is limited, which hinders mixing between populations by larval dispersal alone (Keough, 1989; Wendt, 1996). Larvae are brooded on the colony until they are ready to be released, are nonfeeding, and spend only a few hours in the water column before settling. On the other hand, settlement of these organisms on boat hulls and floating debris allows wide dispersal and mixing between populations separated by long distances.

B. neritina is commonly studied in the laboratory and in field studies because of its abundance and the ease with which larvae can be collected and induced to settle in the laboratory. In recent years, attention has focused on the chemistry of this invertebrate. *B. neritina* produces a series of cyclic macrolides, the bryostatins, which have cytotoxic properties including action against certain cancer cell lines. There are currently 18 different described bryostatins; all have in common the bryopyran ring but vary at two substituent sites (Fig. 1). The structural similarity between the bryostatins and compounds produced by bacteria has led to the proposal that the bryostatins are produced by bacterial symbionts (Anthoni *et al.*, 1990). Bacterial symbionts are borne inside the larvae (Woollacott, 1981); these are γ-proteobacteria named "*Candidatus* Endobugula sertula" (Haygood and Davidson, 1997). These bacteria have not been cultivated; *Candidatus* is a taxonomic status used for bacteria when it is not possible to deposit a type strain (Murray and Stackebrandt, 1995).

Clinical research efforts have concentrated on bryostatin 1,

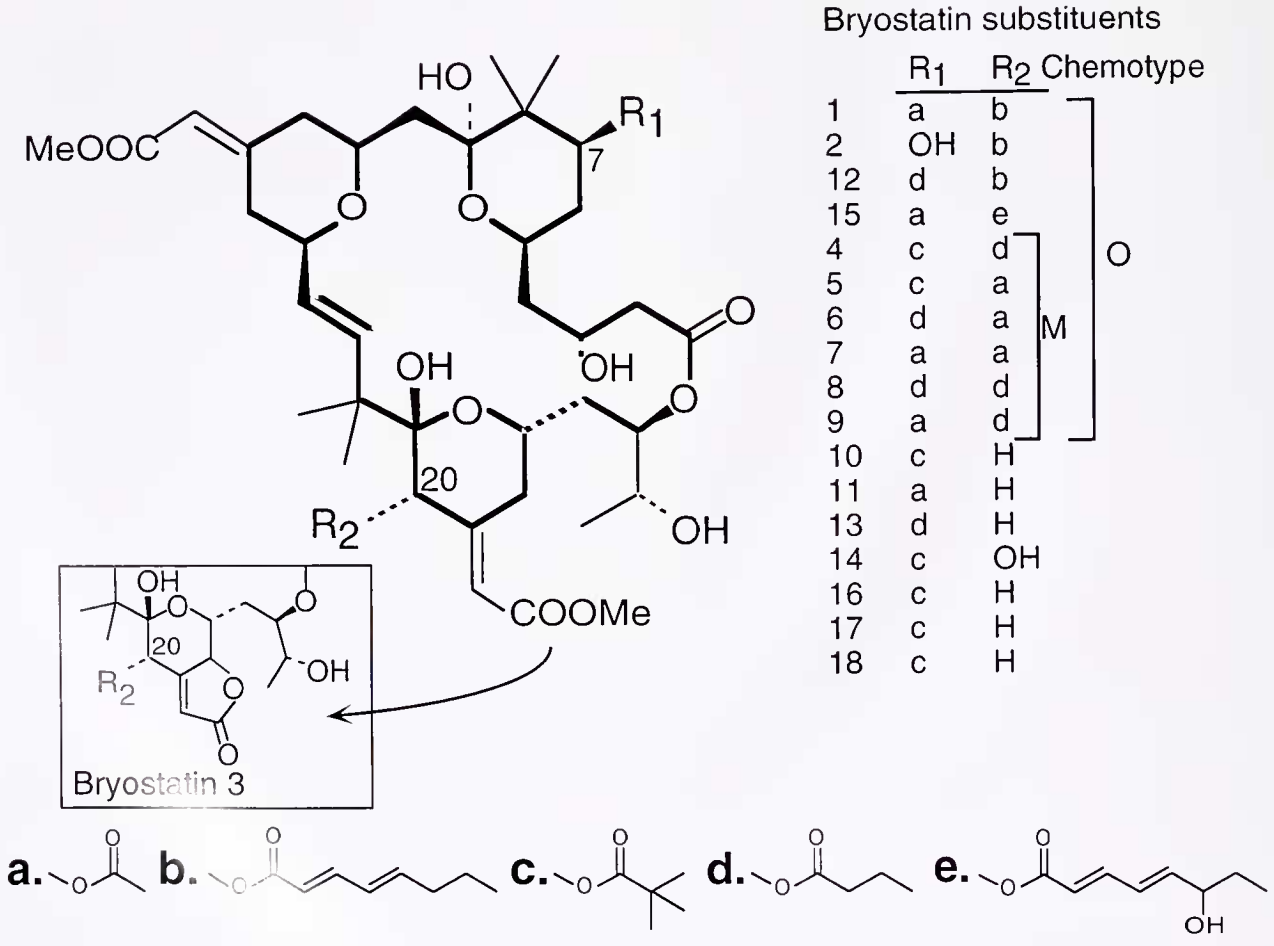

Figure 1. Structure of bryostatins. The bryopyran ring is in bold. The bryostatins of chemotypes M and O are bracketed.

which is in Phase II clinical trials for the treatment of leukemias, lymphomas, melanomas, and solid tumors (Pluda *et al.*, 1996). Pettit and colleagues observed that bryostatin 1 was found in *B. neritina* samples from California, but not from the Gulf of Mexico (Pettit, 1991). In more extensive work in Southern California, Mendola observed that bryostatin 1 is typically found in deeper samples, but is absent from shallow populations (D. Mendola, pers. comm.). We have defined two bryostatin chemotypes, O and M, on the basis of the observations of Pettit and colleagues in California and Gulf of Mexico populations (Pettit, 1991, Fig. 1). Chemotype O (octa-2,4-dienoate) contains bryostatins with an octa-2,4-dienoate ester at C-20 (bryostatins 1-3, 12, and 15) and small amounts of the minor bryostatins 4-9. The presence of bryostatins 1, 2, or 3 is diagnostic of chemotype O. Chemotype M (minor) contains the minor bryostatins 4-9 only, and lacks the octa-2,4-dienoate ester at C-20. The presence of minor bryostatins and the absence of bryostatins 1, 2, or 3 is diagnostic of chemotype M. Because the occurrence of bryostatins 10-18 is not well characterized, they are not included in the chemotype descriptions.

To investigate the relationship between chemotype and genetic variation in *B. neritina*, three characters were analyzed in samples from 13 locations at various depths along the California coast (Fig. 2), and from one Atlantic location (North Carolina): (1) bacterial symbiont small subunit ribosomal RNA gene sequence (SSU rRNA), (2) host bryozoan mitochondrial cytochrome *c* oxidase subunit I gene sequence (CO I), and (3) bryostatin content.

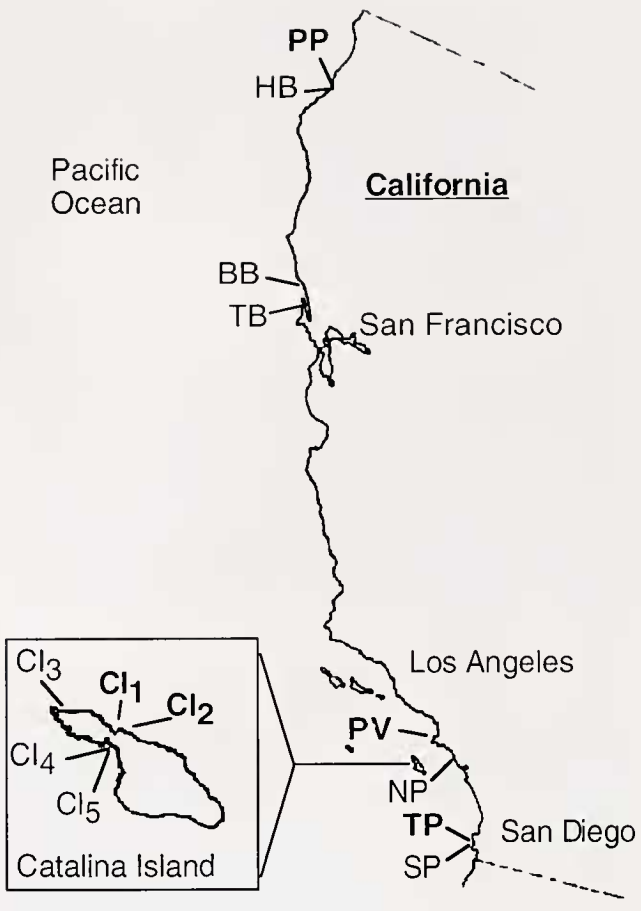

Figure 2. Sampling locations along the California coast. Locations labeled in bold are deeper than 9 m; locations in plain text are shallower than 9 m. Abbreviated site names are designated in Figure 4.

Materials and Methods

Sampling regime

Colonies of *B. neritina* were collected by hand, off docks or subtidally using scuba, along the California coast from Humboldt Bay to San Diego (Fig. 2). *B. neritina* was collected from different depths (ranging from surface to 24 m) at the same location when possible, and at nearby locations in most cases. In addition, one sample was collected in the Atlantic Ocean, from a settling panel array in Beaufort, North Carolina. Samples for chemical analysis were frozen live or dried in the field, then stored frozen at −20° or −80°C. At each site, individual colonies were selected for DNA analysis; these were immediately placed in 95% ethanol in the field and kept cool until stored at −20°C.

DNA extraction, polymerase chain reaction, and sequencing

DNA was extracted from individual colonies by using the QIAGEN QIAamp tissue kit and following the instructions for tissue. To avoid contaminating parent-colony DNA with progeny DNA, only the portions of a colony lacking ovicells, which contain larvae, were used. If necessary, ovicells were removed with forceps before DNA was extracted.

Polymerase chain reaction (PCR), using symbiont-specific primers and conditions described in Haygood and Davidson (1997), was used to amplify about 1060 bp of the "*E. sertula*" SSU rRNA gene. Approximately 710 bp of the bryozoan mitochondrial cytochrome *c* oxidase subunit I gene (CO I) was amplified using primers and conditions described in Folmer *et al.*, 1994. Taq polymerase (Boehringer Mannheim) and included buffer were used for all reactions.

Sequencing was performed using the Applied Biosystems, Inc. (ABI) Prism ready reaction kit FS using guidelines provided by ABI, and reaction products were analyzed on an ABI 373A automated sequencer. The SSU rRNA gene was sequenced using symbiont-specific and universal eubacterial SSU rRNA primers (Haygood and Davidson, 1997; Lane, 1990). The CO I gene was sequenced using the amplification primers. Sequences were aligned in AssemblyLign (Kodak), and differences were counted and com-

pared against the total base pair count to calculate percentage differences. PAUP was used for maximum parsimony phylogenetic analysis (Swofford, 1990), with a transversion cost of 2 relative to transition. Position 365 was excluded from the analysis due to a possible sequencing artifact. A heuristic search was conducted with 10 starting trees created by random addition, and one best tree was found; bootstrap resampling was done 100 times.

*Nhe*I *restriction digest*

A sequence difference in the SSU rRNA gene between two detected strains of "*E. sertula*" allows rapid identification of strain type by digestion with *Nhe*I (see Fig. 3). The *Nhe*I restriction enzyme cuts only the amplified SSU rRNA gene from one strain (type D) into two fragments. The SSU rRNA gene amplification products were cleaned using the QIAGEN QIAquick PCR purification kit, then digested with *Nhe*I (Boehringer Mannheim) and analyzed by agarose gel electrophoresis.

Bryostatin content analysis

Bryostatin content analyses were performed by Dr. M. Koleck of SAIC-Frederick, under contract to the National Cancer Institute. *B. neritina* samples, 50-90 grams wet weight from each sample site, were sent frozen to SAIC-Frederick, where they were lyophilized, extracted, and analyzed for bryostatin using high-pressure liquid chromotography (HPLC) (Schaufelberger *et al.*, 1990). A portion of each dried sample was removed for extraction and HPLC analysis. Subsample size was 5 g except for samples HB1 (4.44 g) and HB2 (3.47 g). A Rainin-Microsorb column (3 μm C-18, 100 $\times$ 4.6 mm I.D.) and corresponding precolumn (15 $\times$ 4.6 mm I.D.) were used for HPLC separations. The system includes a Waters 600 E system controller, a Waters 715 Ultra Wisp sample processor, and a Waters 996 photodiode array detector. The mobile phase was aqueous 78% MeCN, UV, at 1 ml/min. Samples were dissolved in MeOH (2 ml). All sample solutions were filtered through Anotop 0.2-μm filters prior to injection (10 μl).

A bryostatin 1 standard was used to establish a bryostatin 1 standard curve to determine the amount of bryostatin 1 in each sample (data not shown). The National Cancer Institute (NCI) standard method for bryostatin analysis was established on the basis of HPLC retention times, absorbance spectra, and phorbol dibutyrate displacement assays of peaks from many *B. neritina* samples. Co-injections of bryostatin 1, 2, and 3 standards confirmed the validity of the method. The results of extensive analysis of *B. neritina* samples has shown that retention times and absorbance spectra are sufficient to identify bryostatins (David Newman, NCI, pers. comm.). The presence of bryostatins 1, 2, and 3 was determined by the appearance of peaks at established HPLC retention times (~11.3, ~5.2, and ~8.1 min respectively) and confirmed by absorption spectra for these peaks scanned from 200 to 600 nm. The diagnostic UV absorbance peaks for the bryostatins that contain the octa-2,4-dienoate ester are at 230 nm and 265 nm.

The presence of the minor bryostatins was determined, but individual minor bryostatins were not identified. The minor bryostatins (4-11) each have different HPLC retention times and similar absorbance peaks at 230 nm but not at 265. Most of the common minor bryostatin HPLC peaks are observed at ~9.8 min and ~6.5 min retention. If HPLC peaks cannot be confirmed by the absorbance spectra, the bryostatin in question is scored as not detected.

Chemotypes were assigned as follows: if the minor bryostatins were detected in the absence of bryostatins 1-3, chemotype M was assigned. If bryostatins 1-3 were detected, chemotype O was assigned. In chemotype O *B. neritina*, bryostatins 1-3 are found at much higher levels than the minor bryostatins 4-9 (Pettit, 1991). Thus if minor bryostatins are detectable and bryostatins 1-3 are absent, the sample can be assigned to chemotype M with confidence.

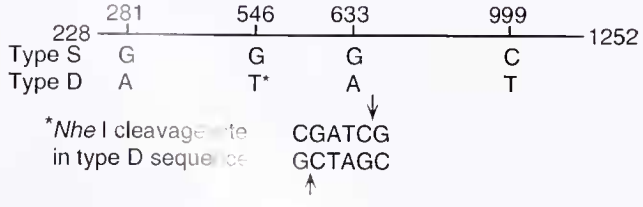

Figure 3. Polymorphic sites in the SSU rRNA gene of "*Endobugula sertula*" strains D and S. The fragment represented results from PCR amplification of the SSU rRNA gene using the symbiont specific primers from Haygood and Davidson (1997). Nucleotide numbers are based on alignment with *E. coli* SSU rRNA gene.

Results

Symbiont strain analysis

The "*E. sertula*" SSU rRNA sequences from populations in Southern California, Northern California, and North Carolina (Atlantic) were identical except for consistent differences at four nucleotide sites within the 1024 bp sequenced from seven locations (Fig. 3). These differences are fixed, not base-paired with each other; they divide the samples into two strains, D (deep) and S (shallow). One of these polymorphisms fell within a restriction enzyme recognition site such that digestion of the SSU rRNA gene amplification product with *Nhe*I could be used to screen individual *B. neritina* colonies for "*E. sertula*" strain (Fig. 3). In Southern California, colonies from sites less than a mile apart were found to have different strains: strain S at sites shallower than 9 m and strain D at sites deeper than 9 m (Figs. 2, 4). In Northern California, colonies containing strain D symbiont were found on the same docks (HB2, Humboldt Bay, Woodley Marina) and shallow rocks

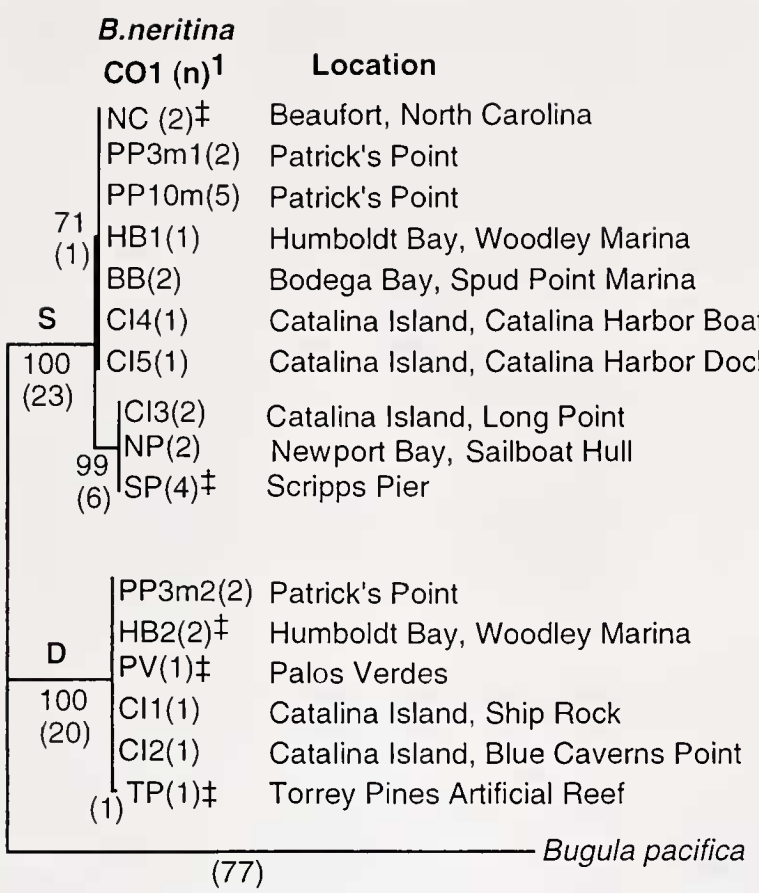

Figure 4. Summary of results. [1]Phylogenetic relationships among *Bugula neritina* CO I genes based on 480 nucleotides. *Bugula pacifica* is selected as the outgroup. Horizontal branch lengths are proportional to the number of steps separating groups. Numbers show the bootstrap support of the labeled nodes. Numbers in parentheses indicate the number of steps between nodes. ‡ 638 bp sequenced on both strands. [2]M, minor bryostatins detected; O, bryostatins 1-3 and minor bryostatins detected; * tentative, based on HPLC retention times and very weak absorbance spectra for bryostatin 3 and minors; ND, bryostatins not detected. [3]"*E. sertula*" SSU rRNA genotype determined by *Nhe*I digestion as shown in Fig. 2. † genotypes confirmed by sequencing.

(PP3m2, Patrick's Point, 3 m) as colonies containing the strain S symbiont (HB1 and PP3m1). In Bodega Bay (BB) and Tomales Bay (TB; not shown in Fig. 4 or 5, sequence data similar to BB) only the strain S symbiont was detected. To date, both strains of "*E. sertula*" have not been found within the same colony of *B. neritina*.

Host bryozoan CO I gene sequence analysis

At most locations sampled, a single "*E. sertula*" strain was detected within the *B. neritina* population. For sequence analysis of the CO I genes, a region of 480 nucleotides was examined. For locations shown to contain a single "*E. sertula*" strain, one or two samples were sequenced, on the assumption that hosts with similar genotypes would contain similar "*E. sertula*" strains. In the case of Northern California, where both symbiont types were detected at the same location, the CO I gene was sequenced from two colonies of each representative type to ensure detection of differences between the hosts. To confirm the accuracy of using the single-stranded CO I sequence, five samples (each from a different site) were sequenced for 638 bp on both strands; all showed similar results. Two distinct lineages (D and S) of *B. neritina* were found. The difference between the two was 8.1% for the 480-bp region (Figs. 4, 5) and 9.1% over 638 bp (data not shown). Additional sequence differences were detected within the type S *B. neritina*, distinguishing two groups that are much more closely related. Both S types were found in Southern California. At Catalina Island, both S_1 and S_2 were present, showing that these two genotypes are present in the same area and at similar depths.

Colonies of CO I genotype D contained "*E. sertula*" strain D, and colonies of CO I genotype S contained "*E. sertula*" strain S.

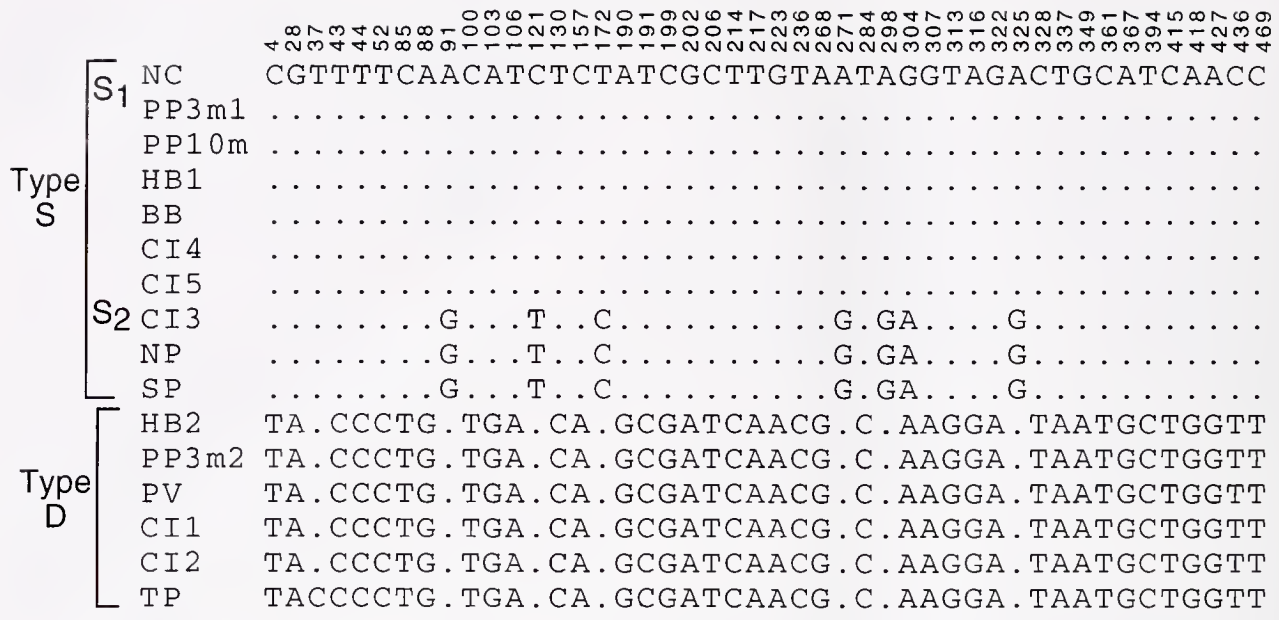

Figure 5. *Bugula neritina* cytochrome *c* oxidase subunit I gene alignment showing only the variable sites. Sequence corresponds to position 1521-1990 in the published *Drosophila yakuba* mitochondrial genome sequence (Clary and Wolstenholme, 1985) with nucleotide position number noted above. The brackets indicate sequences for *B. neritina* varieties type D and S, including the differences within type S as S_1 and S_2.

Bryostatin content analysis

Bryostatin analysis by HPLC showed that bryostatins of chemotype O (bryostatin 1-3) were detected consistently in *B. neritina* collections from deeper than 9 m in Southern California, and the minor bryostatins of chemotype M (bryostatins 4-9) were detected in *B. neritina* from shallower than 9 m (Figs. 2, 4). Several of these populations have been sampled repeatedly for bryostatin analysis for other studies as well as for this paper and have given consistent results.

At sites farthest north, separation of chemotypes by depth was not apparent, but this result must be interpreted with caution because the chemistry data from *B. neritina* taken in the Humboldt area are ambiguous. HPLC peaks indicative of minor bryostatins, but no peaks for bryostatin 1, 2, or 3, were detected in a *B. neritina* sample from deeper than 9 m (PP10m, Patrick's Point, submerged rocks outside of Humboldt Bay); these findings are indicative of chemotype M. Absorbance spectra were weak, but consistent with minor bryostatins. This sample had CO I type S and "*E. sertula*" strain S. One of the dock samples from Humboldt Bay (HB2) yielded HPLC peaks at the correct retention times for bryostatins 1, 2, and 3, and the minor bryostatins, indicating chemotype O. However, the absorbance spectra were too weak (bryostatin 3), or possibly were obscured by compounds in overlapping peaks, to positively confirm the bryostatin identification. For these two Northern California samples, chemotype designations are tentative due to lack of clear confirmation by absorbance spectra.

Consistently, the collections of *B. neritina* with type D CO I genotype and strain D "*E. sertula*" contained the bryostatins of chemotype O. Those samples with genotype S host and symbiont contained bryostatins of chemotype M.

Discussion

In this study we identified two types (D and S), probably species, of the marine bryozoan nominal species *Bugula neritina* that differ in their mitochondrial CO I sequences by approximately 8%. Each type harbors a distinct strain of the bacterial symbiont "*E. sertula*" and has a characteristic chemistry profile. Both of these *B. neritina*/"*E. sertula*" associations produce bryostatins, but only type D contains the additional bryostatins 1-3, characterized by the octa-2,4-dienoate ester at C-20 of the bryopyran ring (Fig. 1). We found that, without exception, the type D *B. neritina* lineage contains strain D "*E. sertula*" and chemotype O bryostatins. The type S *B. neritina* lineage contains strain S "*E. sertula*" and bryostatins of chemotype M (Fig. 4).

In Southern California, the two types of *B. neritina* were found in the same areas but at different depths (transition at about 9 m). In Northern California, colonies containing type D host and symbiont were found on the same docks (Humboldt Bay) and shallow 3-m rocks (Patrick's Point) as colonies containing the type S host and symbiont. This pattern suggests that the two types are separated by an environmental parameter such as temperature, as has been found in some other marine invertebrates (Hellberg, 1998;

Knowlton, 1993). In Southern California, where the two types are separated, surface waters become several degrees warmer in the spring through early fall. In Northern California, however, the surface remains much cooler. Additional research must be conducted before any conclusions can be drawn regarding the cause of the separation. The North Carolina *B. neritina* matches the type S *B. neritina* in California. The predominance of type S on boat docks and hulls and its appearance in both the Atlantic and Pacific indicates that this genotype of *B. neritina* is transported on boat hulls and may have a worldwide distribution mediated by human transport. The type D genotype, however, has a more restricted distribution that relies on larval dispersal and promotes divergence between widely separated populations. Additional samples from deep locations in the Atlantic and other areas will be valuable in confirming this hypothesis. The type S may have a broader temperature tolerance or other characters, such as better pigment protection from UV, that allow it to survive conditions it may experience on boat hulls. Taken together, these data suggest that two sympatric species of *B. neritina* exist in California.

A serendipitous experiment provided evidence that the type D *B. neritina* does not colonize shallow habitats in Southern California even if type D larvae are introduced there. *B. neritina* is a normal inhabitant of the Scripps Pier, La Jolla, California. Over a period of 3 years, larvae from Palos Verdes parent stock (consistently chemotype O and type D genotype) collected at about 16.5 m have been poured into the water around the pier as a consequence of aquaculture research. Cumulatively, these releases, each lasting 2 weeks and taking place three or four times yearly from spring through fall, put hundreds of thousands of larvae into the water. In November 1997, about a month after the end of the summer season of larval release, samples were taken from the Scripps Pier pilings in an attempt to detect any type D *B. neritina* that may have become established over the 3-year period. At the time, the only *B. neritina* present were located at the bottoms of the pilings (4.5-7.5 m). The colonies were small (5 cm tall), which matched expected colony size for the settlement dates of the recently released larvae. Collections were taken from each northern piling along a transect from just below the location of release out to the end of the pier. Five colonies from each of the six pilings sampled were tested for symbiont genotype. Strain D symbionts were not detected in any of the samples. All the sequences and *Nhe*I digests of the SSU rRNA "*E. sertula*" gene taken from previous pier samples over 3 years have been strain S. The host bryozoan CO I gene was sequenced from two individuals, and these sequences matched type S. Bryostatin analysis of each of the pier samples and of previous collections revealed only the bryostatins of chemotype M, consistent with the genotype found. These results suggest that the type D *B. neritina* is not able to establish a major population on the pier.

The finding that chemotypes O and M occur in distinct lineages of *B. neritina* indicates that there is a genetic basis for patterns of bryostatin synthesis. Previous experimental work has shown that bryostatins do not accumulate from the diet (Thompson and Mendola, 1993), but did not rule out the influence of other environmental factors in determining the types of constituents used in bryostatin synthesis. A type D shallow sample (HB2) probably contains chemotype O bryostatins (by HPLC retention times), whereas a type S sample at 10 m (PP10m) does not, and is probably chemotype M. If so, these samples strengthen the argument that bryostatin substituents are determined by genetic capabilities and not by environmental factors associated with depth. It is possible that the chemotype differences are due to the different strains of "*E. sertula*" in the two types, to a difference in the host bryozoan, or to an additional unknown bacterium. In any case, one *B. neritina*/"*E. sertula*" mutualism is capable of making the octa-2,4-dienoate substituents of bryostatins 1-3, 12, and 15, and the other is not. It is also possible that there are additional genotypes of *B. neritina*/"*E. sertula*," and new bryostatins may be discovered in areas that remain to be sampled.

In some cases the consistent re-isolation of natural products from marine invertebrates has been problematic, possibly because of difficulty in distinguishing closely related species or subspecies. On the other hand, geographic variation in bioactive compounds from a few bryozoans has been attributed to environmental factors (Christophersen, 1991). The present study provides evidence that invertebrate sibling species can contain different natural products, and it illustrates that distinguishing variants is important to ensure consistency in natural product chemistry. Variations in secondary metabolite chemistry should be included among the reasons, discussed by Knowlton and Jackson (1994), that proper taxonomy is critical to understanding the diversity, evolution, and ecology of a system. Molecular characterization provides a powerful tool for this endeavor. The chemistry, chemical ecology, and biology of variants may differ slightly but significantly, as in the case of *B. neritina*, where the genotypes are indistinguishable in the field, but one produces the commercially important compound and the other does not. Moreover, these chemical differences may determine the predators to which the colonies are susceptible. It is possible that these or other subtle chemical differences affect chemical defense and thus influence the ecology of the bryozoan.

Genbank Accessions #s: PP3m1, AF061429; PP3m2, AF061430; PP10ml, AF061431; HB1, AF061427; HB2, AF061428; BB, AF061426; C11, AF061417; C12, AF061418; C13, AF061419; C14, AF061420; C15, AF061421; PV, AF061422; NP, AF061423; TP AF061424; SP, AF061425; NC, AF061432; *B. pacifica*, AF061433.

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